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Development of electrochemical method to detect bacterial count, *Listeria monocytogenes*, and somatic cell count in raw milk

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ABSTRACT

Bacterial count, *Listeria monocytogenes* detection and somatic cell count (SCC) are generally recognized as indexes of raw milk quality which could affect the price and the shelf-life of dairy products. This research was aimed at developing an easy, low-cost and rapid quantitative electrochemical detection method for measuring the three indexes simultaneously. Through the electrochemical sensor, bacterial count or *L. monocytogenes* detection was determined by evaluating the electric-current originated from microbial activity. SCC was determined by measuring current signal produced by the expression of lactate dehydrogenase (LDH) activity, a biomarker also for diagnosing mastitis. The research indicated that the appropriate conditions for applying the biosensor to bacterial count detection ranging from 10^2 to 10^8 CFU/ml within detection time (DT) of 1–10 h are 35 °C at 1 V and that the optimal conditions for *L. monocytogenes* detection are 37 °C at 1 V, under pH 9.4, associated with a Fraser selective broth. The optimal conditions for SCC detection ranging from 350 to 780 thousand SCC/ml ($R^2 = 0.907$) within 60 s (DT) are 42 °C at 0.5 V, under pH 9.0. The biosensor is shown to be a promising approach for the detections in raw milk.

1. Introduction

For production of high-quality dairy products, the composition and properties of raw milk are major concerns. Although the advanced progress in developing genetics, feeding systems, housing and milking conditions, there is a remarkable increase in a group of multi-factorial diseases in most of the high-producing dairy farms. Among these diseases, mastitis is still the most economically important one, which might lead to significant milk losses, therapeutic expenses [1], changes in milk sanitation, quality as well as properties [2]. In general, clinical mastitis can be remarked by the farmer, however subclinical infections are difficult to be recognized, which result in drastic milk losses, increased somatic cell count (SCC), and pathogen appearance in the secretion. Subclinical mastitis can only be detected by analyzing the inflammatory components and pathogens in the milk [3]. MacMillan et al., has found that in the case of clinical mastitis with high SCC, the depressive effect on milk production will be mostly apparent [4]. As a result of inflammation, the higher SCC in raw milk can be deduced that the udder is infected more gravely [5]. Hence detecting SCC effectively in raw milk would be a critical inspection point for identifying the quality [6]. Considering SCC measurement in milk, currently the most important sensors available in practice are approaches for evaluating EC (electrical conductivity), LDH, color and image, SCC [7,8]. However, the sensors are usually less sensitive or relatively expensive. Nowadays, automatic milk somatic cell counting instruments is employed frequently in dairy factory, though they possess high accuracy and high sensitivity, the cost of the reagents and instrument is not affordable by the dairy farmers.

Currently, a number of methods have been developed as well for bacterial cells detection [7]. Although the oldest detection technique, culturing and plating method, remains still the standard method for detecting pathogenic bacteria, they are excessively time-consuming and thus are obviously inconvenient in many industrial applications [9]. Numerous rapid alternated techniques have been developed in recent decades, including ATP estimations [10], direct epifluorescent filter technique [11], methylene blue reduction method [12]. Besides, impedance measurement [13], polymerase chain reaction (PCR) [14], optical sensor [15], flow-cytometry [16], microscopy [17], immunology based assay [18], etc., have also been studied. Though some of the methods are fast, most of the others show long response time and are either time consuming, complicated, expensive or low selective in general. A novel series piezoelectric quartz crystal (SPQC) sensor for bacterial detection in raw milk was recently investigated, which was cheap and compact, while a greater frequency drop and fluctuation would be

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encountered in case of higher initial bacterial concentrations [19]. The electrochemical approach is promising for its specific, rapid and widely applicable properties in cells detection with an appropriate selective media [9,20]. The electroactive species generated during the enzymatic reaction can be measured by amperometric sensors [21], moreover, which have attractive advantages over other analytical systems in that they can operate in colorful and turbid media [22], offer repetitive assays with specificity and sensitivity using miniaturizable, robust, and inexpensive devices [23,24].

Among the common pathogens that should be paid attention to is *Listeria monocytogenes*, which is one of the most hazardous microbes in contaminated foods. It can even cause listeriosis in human bodies and has been considered as an important foodborne pathogen notable for both its persistence in food manufacturing environments and especially dire consequences of infection with a high mortality rate. [25].

Our study developed a simple, rapid and low-cost detection system by means of an alternative amperometric sensor. The change in electrochemically active metabolites production from microorganisms could be monitored to calculate bacterial count (and *Listeria monocytogenes*). SCC was detected by measuring the current signals resulting from the expression of LDH activity. The objectives were to evaluate the effectiveness of the electrochemical sensor for determination of the bacterial count, *Listeria monocytogenes* and SCC in raw milk.

2. Experimental

2.1. Reagents and materials

Nutrient broth, plate count agar (PCA) were obtained from BD (Becton Dickinson and Company, Maryland, USA), *Listeria* fraser broth base and supplement were obtained from Merck (Merck Chemicals, Germany). Culture of *Escherichia coli* was obtained from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan) and cultures of *Listeria monocytogenes, Staphylococcus aureus*, *Salmonella typhimurium* were obtained from Tunghai University (Taichung, Taiwan). All the cultures were inoculated into nutrient broth and incubated at 37 °Covernight, aliquots of the dilutions were spread streakily on pour plates and incubated at 37 °C for 24 h for enumeration. All colonies were counted after 24 h of incubation.

2.2. Plate counting method

Raw milk was diluted with sterilized water for preparing serial decimal dilutions (10^{-1}–10^{-8}). 0.1 mL samples of the raw milk at various dilutions were added to plate containing PCA(45–50 °C). Each dilution was prepared with three replicates. After being blended and solidified, the prepared samples were immediately incubated at 35 °C. Then 25–250 colonies of bacteria were transferred to colony counter (SK-20162, Shin Kong Industries, New Taipei City, Taiwan) for enumeration (CFU/ml).

2.3. Electrochemical measurements

Amperometric measurements were performed using an electrochemical analyzer (model CHI 802B, West Lafayette IN, USA) linked with an uninterruptible power supply (UPS), which were performed using a conventional two-electrode system, with a Pt electrode as working electrode. Input and output signals from the potentiostat were coupled to a personal computer (1.8 GHz Pentium microprocessor).

2.4. Instrumentation

Amperometric sensor was constructed by a laboratory built potentiostat. Input and output signals from the potentiostat were coupled to a PC (Pentium 1.8 GHz) using a peripheral interface card (AT-MIO-16E, National Instruments, Austin, TX, USA), which consisted of a 16-channel analog-to-digital (A/D) converter (12 bit) and a 2-channel digital-to-analog (D/A) converter (12 bit). Data display and recording were programmed using the LabVIEW 7.1 software package (National Instruments, Austin, TX, USA). All measurements were taken with a two-electrode system using a pair of planar platinum strips (Malthus, C6243; Crawleg, UK), which were performed at the optimal condition depending on the experiments.

2.5. Amperometric measurement procedures

The protocol of procedure was carried out essentially following the method described by Lee et al. [26]. The total sample volume was 1 mL for each measurement. Input and output signals from the potentiostat were coupled to a PC and the voltage output, data display, and current change were recorded as mentioned before. The detection time (DT) was plotted against the microbial populations determined by the plate count method to make calibration graph. Microbial concentration in the dilutions was confirmed by plate counting method and the electrochemical method, data from plate count method being as a control.

2.6. Detection of bacterial counts

Raw milk was sterilized by autoclaving at 121 °C for 15 min and conserved at 4–8 °C. Then each 0.9 ml of the aseptic milk was blended with 0.1 ml of raw milk in the reaction cell containing Pt electrode as working electrode. The optimal voltage was assayed and input in the electrochemical measurement system. The optimal temperature was selected and set by a circulating water bath associated with the system. DT measured for each assay was loaded into the calibration graph of DT against microbial density to calculate the bacterial counts after being multiplied by the dilution factor.

2.7. Detection of Listeria monocytogenes

To isolate and identify *Listeria monocytogenes* in raw milk, the assay was realized by using electrochemical method associated with a Fraser selective broth. The common bacteria: *Salmonelloses typhimurium*, *Escherichia coli*, and *Staphylococcus aureus* were inoculated as background controls. The optimal pH value was adjusted to 9.4 by adding 0.1 M Lactic acid. Each sample was prepared by mixing 0.1 ml of microbial culture with 0.9 ml of aseptic milk, which was blended with Fraser broth at a ratio of 1:0.7 in the reaction cell. Thereafter the electrochemical detection system was performed at 37 °C, 1 V and the resulting current response was employed to calculate the growth of the microbes.

2.8. Detection of somatic cells counts

A single tube system was employed in the electrochemical measurement. 75 mM Lactate and 5.5 mM NAD^+(Nicotinamide adenine dinucleotide) were added as substrates. An aliquot (20 ml) of raw milk sample was separated by centrifugation during 20 min at 20 °C into 3 fractions: butterfat, milk serum, and casein proteins phases respectively. Then an aliquot (1 ml each) of the middle portion (milk serum containing LDH) was transferred into the reaction cell to react with the substrates. After the optimized experimental condition was determined, the electrochemical sensor was performed at 42 °C, 0.5 V, with an adjustment of pH value to 9.0 ± 0.1.